

Effect of apical root-end filling materials on gingival fibroblasts

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Abstract

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Aim To determine the influence of root-end filling materials on specific cellular responses of gingival fibroblasts (GF).

Methodology The reactions of cells in contact with mineral trioxide aggregate (MTA), amalgam and a chemically inert titanium alloy were determined based on the assessment of prostaglandin (PGE₂) release with and without arachidonic acid stimulation, protein and lactate synthesis, and cell proliferation. Cells cultured without test materials served as controls (100%).

Results The fibroblasts showed a highly significant decrease in protein synthesis when in contact with amalgam ($61.8 \pm 13.6\%$); MTA ($91.2 \pm 5.9\%$) and titanium ($92.4 \pm 4.7\%$) had little effect on this parameter. The rate of cell proliferation in contact with MTA ($98.0 \pm 1.6\%$) and titanium ($97.9 \pm 7.4\%$) was only slightly influenced and showed similar values to that of

the controls after 96 h of incubation. On the contrary, a significant and continuous reduction in the rate of cell proliferation was observed for cells in contact with amalgam ($61.0 \pm 2.5\%$) after 96 h. No significant increases in lactate synthesis values were registered for any of the materials (MTA $101.8 \pm 1.7\%$, titanium $94.8 \pm 8.6\%$ and amalgam $105.8 \pm 10.3\%$). There was a significant decrease in PGE₂ synthesis potential when cells were in contact with amalgam ($85.2 \pm 3.5\%$). In comparison to the controls, titanium and MTA resulted in an elevated level of cellular PGE₂ synthesis (titanium: $131.6 \pm 19.1\%$; MTA: $147.3 \pm 18.9\%$). The cell cultures stimulated with arachidonic acid (10^{-5} M) showed no significant differences with any material (MTA: $88.8 \pm 17.6\%$, titanium: $97.6 \pm 14.4\%$, amalgam: $85.5 \pm 16.8\%$).

Conclusions MTA demonstrated cellular responses similar to those of titanium. Amalgam showed an irritation rate higher than that of MTA and titanium.

Keywords: amalgam, gingival fibroblasts, MTA, prostaglandin release, protein synthesis, titanium.

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Introduction

Dental materials may have a significant influence on the tissues of the orofacial system because their potentially toxic ingredients can initiate irritation of the neighbouring tissues, may interfere with the healing process or may cause allergic reactions. The materials used for endodontic procedures must be chosen with care so that potential periapical reactions can be avoided or reduced. The tissues surrounding the apex of a tooth root are

exposed not only to root-canal filling materials used during root-canal treatment, but also to materials placed during endodontic surgical procedures including the repair of perforations. Ideally, endodontic materials should be biocompatible.

Mineral trioxide aggregate (MTA) is a promising material, recommended for pulp capping, sealing of perforations and apexification; several studies have reported its biocompatibility (Torabinejad *et al.* 1995, Mitchell *et al.* 1999, Keiser *et al.* 2000). The potential interaction of MTA with specific cellular parameters such as prostaglandins or its effects on cellular lactate production have also been examined (Willershausen *et al.* 2000).

Amalgam has been used as a root-end filling material for many years, and it is still used as a cost-effective

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material in conservative dentistry. Findings such as allergic reactions, lichenoid mucosal reactions linked with the use of amalgam or its potential effects on the immune system emphasize the controversy of its clinical use.

In vitro studies enable the investigation of the reactions of cell functions to specific influences. The reaction patterns of human diploid cells are comparable to those of the oral mucosa, thus allowing the investigation of cell lines in the absence of additional interfering influences.

The aim of this study was to investigate several essential metabolic cellular responses of gingival fibroblasts (GF) exposed to MTA, amalgam and a chemically inert titanium alloy by means of cell proliferation, protein and lactate synthesis and prostaglandin (PGE₂) release assessment.

Materials and methods

Test materials

Samples of MTA (ProRoot™ MTA, Dentsply Maillefer, Ballaigues, Switzerland), amalgam (Dispersalloy, Dentsply Caulk, Milford, DE, USA; 49.8% mercury amalgamated with 50.2% alloy powder, setting time 72 h; 100% alloy powder: silver 69.3%, tin 17.9%, copper 11.8%, zinc 1.0%) and a titanium alloy (Straumann, Waldenburg, Switzerland; titanium 98.9%, iron 0.5%, oxygen 0.4%, carbon 0.1%, nitrogen 0.05%, hydrogen 0.015%) were incubated with GF for up to 9 days. Cells cultured without test materials served as controls (100%).

The MTA (10 mg) and amalgam (100 mg) samples were prepared according to the instructions of the manufacturers. MTA was allowed to set for 24 h under UV light prior to the culturing procedures. The titanium (100 mg) and the amalgam samples were stored in 70% alcohol and exposed to UV light for 24 h to prevent contamination of the cell cultures.

Cell cultures

Gingival fibroblasts were cultured from biopsies obtained during tooth extraction from healthy patients. Fibroblasts between the 5th and the 7th passages were used in this study. Standard protocols were followed in establishing and maintaining the cultures. The culture medium DMEM/F12 (Dulbecco's modified Eagle's basal medium) was supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. The cells were subcul-

tured by trypsinization as described in previous studies (Briseño & Willershausen 1990).

Cell viability

Cell viability and cell number were determined by a trypan blue exclusion test, using a Fuchs–Rosenthal counting chamber. Cell viability was determined through cytosolic esterase activity measurement by means of fluorescent dyes for DNA staining (calcein acetoxymethylester: excitation 485 nm, emission 530 nm; ethidium homodimer-1: excitation 485 nm, emission 600 nm), according to the manufacturer's recommendations (Molecular Probes, Leiden, The Netherlands). Fluorescent activity was measured using the Fluoroscan Ascent (LabSystems, Helsinki, Finland).

Prostaglandin E₂ assay

PGE₂ from the cultured cells was determined by incubating the test materials in 96-multiwell microtitre plates. Layering was performed at a density of 15 000 fibroblasts well⁻¹. Subsequently, the cells were incubated at 37 °C for 30 min, and separated by centrifugation for 5 min at 800 *g* and 4 °C. The supernatants were removed and stored at –20 °C until analysis. In additional test series, the cells with and without the test materials were incubated in a cell culture medium containing arachidonic acid (10⁻⁵ M, Biomol, Hamburg, Germany) for 30 min. PGE₂ was determined from the supernatants with a highly sensitive and specific competitive enzyme immunoassay (Schäfer *et al.* 1999) using PGE₂ monoclonal antibodies (*n* = 4; clone E2R1).

Protein and lactate synthesis

To determine the influence of the test materials on cellular protein synthesis and lactate concentrations, the materials were placed in 24-multiwell chambers (Becton Dickinson, Plymouth, UK) and seeded with fibroblasts at a density of 30 000 cells well⁻¹. The cells with and without the test materials were incubated for 6 days, according to standard culture techniques. The protein content of the cells (mg protein well⁻¹) was assayed spectrophotometrically with the Bicinchoninic acid (BCA) test (*n* = 5; Pierce Chemical Company, Rochford, Illinois, USA). The lactate concentration was determined by four-fold measurements over a total period of 9 days using a commercially available UV test (L-lactic acid test), according to the instructions of the manufacturer (Boehringer, Mannheim, Germany).

Cell proliferation assay

A Redox indicator system (Alamar Blue, Alamar Biosciences Inc., Sacramento, CA, USA) was used to investigate the proliferation activity of GF with and without the test materials. Measurements (Ahmed *et al.* 1994) were performed with a fluorometer (Fluoroscanner Ascent, Labsystems, Helsinki, Finland) at an excitation and an emission wavelength of 538 and 600 nm, respectively, over a period of 96 h ($n = 6$).

Statistical analysis

Descriptive statistics included mean and standard deviations for each measured parameter. The data was analysed by the Kolmogoroff–Smirnov and the Kruskal–Wallis tests at a probability level of $\leq 5\%$.

Results

The control fibroblasts after 30 min of incubation showed a PGE₂ release of 186.5 ± 18.9 pg well⁻¹ (100%). The PGE₂-values of the cells in contact with MTA and titanium showed a release of 147.3 ± 18.9 and 131.6 ± 19.1 %, respectively. In contrast, a significant reduction of PGE₂ ($85.2 \pm 3.5\%$) was observed after direct contact with amalgam (Fig. 1). The PGE₂ release of the control cells following arachidonic acid stimulation was 448.5 ± 120.1 pg well⁻¹, and this was used as a reference value (100%). In comparison, the PGE₂ release after stimulation with arachidonic acid recorded for the GF in

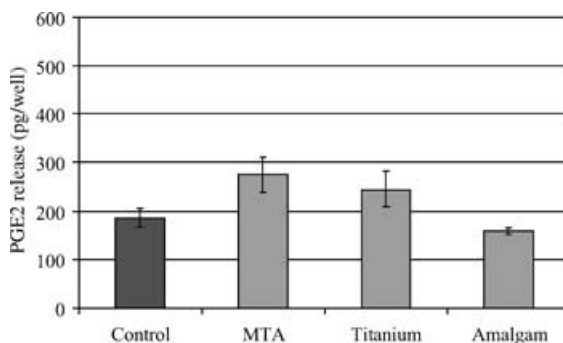


Figure 1 Prostaglandin (PGE₂) release by gingival fibroblasts (GF) exposed to mineral trioxide aggregate (MTA), titanium and amalgam (mean \pm SD). The measured values were statistically higher (MTA and titanium) and significantly lower (amalgam) when compared to the results obtained with the controls, reflecting a PGE₂ release stimulation with titanium and MTA and a PGE₂ synthesis reduction with amalgam.

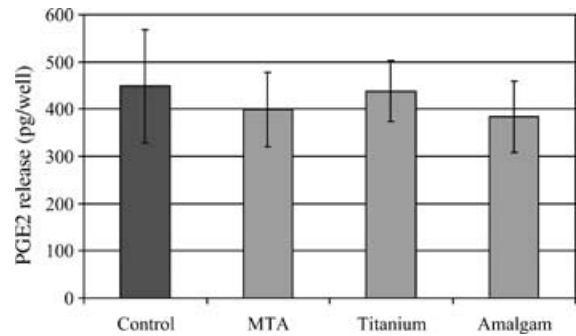


Figure 2 Prostaglandin (PGE₂) release through arachidonic acid stimulated gingival fibroblasts (GF) in contact with mineral trioxide aggregate (MTA), titanium and amalgam (mean \pm SD). No statistically significant differences in comparison with the controls were observed.

contact with titanium was $97.6 \pm 14.4\%$, with MTA $88.8 \pm 17.6\%$ and with amalgam $85.5 \pm 16.8\%$, and the respective control cells showed no significant differences (Fig. 2).

Cellular protein values of 0.1001 ± 0.0079 mg mL⁻¹ were recorded for the control fibroblasts after 6 days of incubation (100%). In all cases, the incubation of the cells with the test materials led to a statistically significant reduction in protein synthesis. However, only slight differences were observed between the control cells (100%) and the fibroblasts incubated with titanium ($92.4 \pm 4.7\%$) and MTA ($91.1 \pm 5.9\%$). The GF showed a substantial reduction when in contact with amalgam ($61.8 \pm 13.6\%$; Fig. 3).

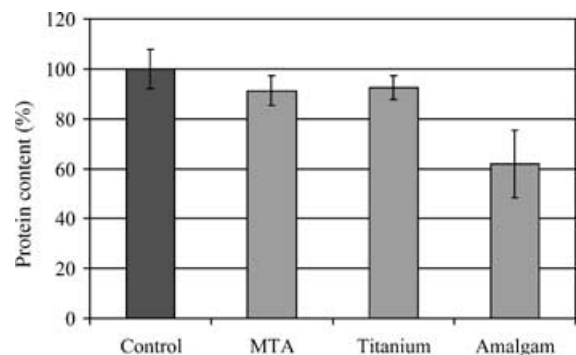


Figure 3 Gingival fibroblast protein synthesis in contact with the tested materials after an incubation time of 6 days (mean \pm SD). A statistically significant protein synthesis reduction in comparison with the controls was observed with all three materials. The cell protein synthesis decrease with titanium and MTA was, although statistically significant, markedly less when compared to that with amalgam.

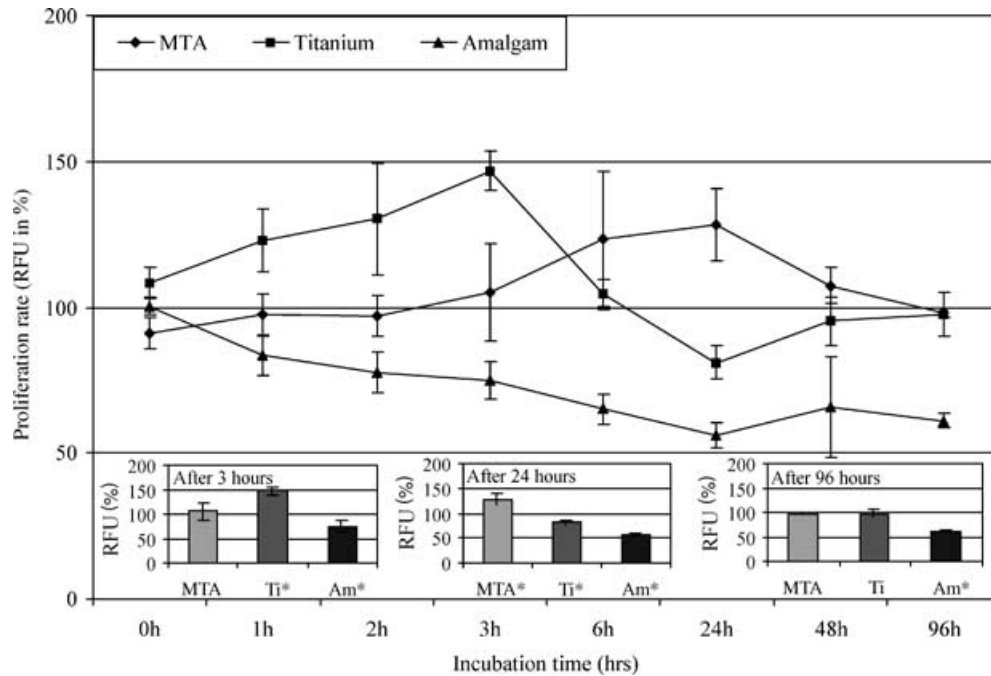


Figure 4 Proliferation rate of gingival fibroblasts (GF) incubated with mineral trioxide aggregate (MTA), titanium and amalgam (RFU: relative fluorescence units, mean \pm SD). The bar graphs depict the proliferation rate differences after 3, 24 and 96 h of culturing. The control cultures were used as reference (100%) Asterisk (*) shows significant differences between the controls and the materials.

The GF showed no significant differences in lactate concentration when compared with the controls (100%) after 9 days of incubation with MTA ($101.8 \pm 1.7\%$), titanium ($94.8 \pm 8.6\%$) and amalgam ($105.8 \pm 10.3\%$).

The short-term measurements (3–6 h) of the proliferation rate observed with MTA rose to $123.6 \pm 23.1\%$ after 6 h in comparison to those observed with the controls (100%). GF incubated with titanium showed a proliferation rate increase to $146.8 \pm 6.9\%$ after 3 h, followed by a reduction to $104.4 \pm 5.1\%$ after 6 h. At the same measurement point, the proliferation rate of the cells incubated with amalgam was $65.1 \pm 5.3\%$ compared to those incubated with the controls. The 24-h proliferation rate with MTA remained relative stable ($128.5 \pm 12.4\%$), whilst $81.1 \pm 5.8\%$ was observed with the titanium samples. The proliferation rate of cells in contact with amalgam was significantly reduced to $55.9 \pm 4.4\%$. The long-term proliferation rates (96 h) when in contact with MTA ($98.0 \pm 1.6\%$) and titanium ($97.7 \pm 7.4\%$) were similar to the control values (100%). On the contrary, the cell proliferation rate in contact with amalgam ($61.0 \pm 2.5\%$) was significantly lower compared to that in contact with the controls (Fig. 4).

Discussion

The present study was designed to determine the influence of different root-canal filling materials on gingival fibroblast cultures. The parameters investigated were protein and lactate synthesis, cell proliferation and PGE₂ release of GF.

The prostaglandin mediator function and its biological significance are inherent to its broad and varied metabolism influence (Schäfer *et al.* 1996, 1999). The different reactions of PGE₂ could explain the proliferation rate promotion (Sauer *et al.* 1998).

Rudnick *et al.* (2001) demonstrated that liver cell regeneration is influenced significantly when the prostaglandin release enzymes cyclooxygenase-1 and -2 (COX-1 and -2) are inhibited. The inhibition of COX-2 inhibited regeneration partially, whereas the inhibition of COX-1 tended to delay regeneration. Furthermore, the same authors reported that prostaglandin signalling is required during cell regeneration. Savla *et al.* (2001) suggested that the specific inhibition of COX-1 and -2 produced a dose-dependent cell wound closure inhibition through airway epithelial cells, whereas the addition of PGE₂ reduced the inhibition of COX-1 and -2, and cell

wound closure was stimulated in a dose-dependent manner. On the contrary, extremely high prostaglandin levels are present in certain inflammatory processes such as periodontitis and are considered as inflammatory reactions (Arai *et al.* 1995). Takayama *et al.* (1996) showed that, in comparison to teeth without periapical radiolucencies, teeth with periapical radiolucencies had increased PGE₂-values. Even during root-canal treatment, the periapical tissues showed noticeable changes in their PGE₂-values (Shimauchi *et al.* 1997). Prostaglandin not only plays an important role during the inflammatory processes but is also considered to be essential for cell regeneration, proliferation and growth processes.

The GF showed increased PGE₂-values in the presence of titanium and MTA. These PGE₂ concentrations pointed to a cell regenerative capability and growth stimulation. The increase associated with MTA was nominally higher in comparison to that with titanium, but no significant differences were found. However, contact of amalgam with GF triggered a reduction in PGE₂ synthesis. This reduction is an expression of the interference with PGE₂ synthesis of cells, and therefore can be considered as an indication of a disturbance in this cell function.

The maximal physiological PGE₂ release of GF can be stimulated with arachidonic acid. The control cell values of PGE₂ measured after arachidonic acid stimulation, which were higher than 240% in comparison to the non-stimulated controls, were not achieved with any of the materials. The PGE₂ release after arachidonic acid stimulation demonstrated only marginal differences between the three materials and controls.

Titanium is widely used in dentistry as an alloy for dental implants, as posts or to increase the root length; therefore, it was included in this research to have a comparison with a clinically established material, which is implanted in similar tissues as root-end filling materials. Titanium is known to produce only limited changes in cell proliferation activity (Peltola *et al.* 1992). Wang *et al.* (1996) observed a reduction in T- and B-cell proliferation following contact with titanium. Thus, the interaction between titanium and interleukin (IL)-2 or IL-6 produced by T cells exerts an effect on regulator molecules of major importance in proliferation, activation, differentiation and maturation processes, as well as in the control of acute phase reactions. Other cell culture (endothelial cells) studies (Wataha *et al.* 1995, Kim *et al.* 1997, Wang & Li 1998) have not reported toxic reactions, cell number alterations, cell lysis, decolonization or cell growth disturbances with titanium. Most studies report

positive findings concerning the biocompatibility of MTA (Torabinejad *et al.* 1995, Keiser *et al.* 2000, Osorio *et al.* 1998). Zhu *et al.* (2000) noted adequate adhesion of human osteoblasts on MTA samples, and Koh *et al.* (1998) detected the stimulation of IL-1a, IL-1b and IL-6 production, suggesting that MTA may be a biologically active substrate.

In this study, titanium produced a temporary proliferation in cells. However, the total proliferation rate (96 h) was similar to that of the controls, showing no cell irritation. The GF in contact with MTA maintained a cell proliferation potential similar to that of the controls and titanium after 96 h of observation. The reduction of the cellular proliferation rate when in direct cell contact with amalgam to approximately 60% compared to that with the control reflected a clear interference with the growth potential of the GF. Peltola *et al.* (1992) were able to show a similar reduction. Tai & Chang (2000) were able to demonstrate similar growth interference.

The protein synthesis potential of the cells in contact with titanium and MTA was statistically significantly lower in comparison to that with the controls. However, the values were over 90% in comparison to the controls (100%) and can be considered to lie within a physiological range and not as a marker for cell irritation. The cell irritations caused by amalgam resulted in a clear reduction of the protein synthesis, which was almost 60% compared to the control.

The cytotoxicity of amalgam is controversial (Torabinejad *et al.* 1995, Zhu *et al.* 2000). The cell cultures investigated in this study were markedly inhibited by amalgam. Its reduced metabolic action, which could be observed through a significantly decreased protein synthesis, cell proliferation rate and PGE₂ synthesis, demonstrated a less favourable reaction of these cell types in comparison to those of the cells incubated with titanium and MTA. Heavy metal ions such as mercury or copper and zinc ions are capable of reacting with groups of hydrolyzed proteins such as SH groups, thus leading to protein denaturation and enzyme inhibition (Ahmad & Stannard 1990). The potential direct influence of mercury ions (Hg²⁺) on the cellular metabolism was also discussed (Liu *et al.* 1992).

The cellular lactate concentration did not increase after incubation with MTA, titanium and amalgam. These values showed that the direct contact of the materials with GF did not cause a cell irritation severe enough that the lactate concentration of the cells, which indicates an anaerobic metabolism, was influenced.

Conclusion

The metabolic activity of GF in contact with titanium or MTA showed a similar reaction pattern. Because of their prostaglandin and proliferation stimulation, these materials had a positive effect on the growth of GF with their unchanged protein and lactate values. Amalgam resulted in the inhibition of the cellular protein and prostaglandin synthesis as well as cell proliferation, and therefore demonstrated its harmful influence on the GF. Based on the cell culture methods and procedures used in the present study, MTA and titanium did not cause PGE₂-related reactions or irritation of the central cellular metabolism. Based on the present study, the use of amalgam as a retrograde root-canal-filling material should be avoided.

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References

- Ahmad R, Stannard JG (1990) Mercury release from amalgam: a study *in vitro* and *in vivo*. *Operative Dentistry* **15**, 207–18.
- Ahmed SA, Gogal RM, Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes. An alternative to [³H]thymidine incorporation assay. *Journal of Immunological Methods* **170**, 221–4.
- Arai H, Nomura Y, Kinoshita M *et al.* (1995) Response of human gingival fibroblasts to prostaglandins. *Journal of Periodontal Research* **30**, 303–11.
- Briseño MB, Willershausen B (1990) Root canal sealer cytotoxicity on human gingival fibroblast. Part I. Zinc oxide-eugenol-based sealers. *Journal of Endodontics* **80**, 483–6.
- Keiser K, Johnson CC, Tipton DA (2000) Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. *Journal of Endodontics* **26**, 288–91.
- Kim TI, Han JH, Lee IS, Lee KH, Shin MC, Choi BB (1997) New titanium alloys for biomaterials: a study of mechanical and corrosion properties and cytotoxicity. *Bio-Medical Materials and Engineering* **7**, 253–63.
- Koh ET, McDonald F, Pitt-Ford TR, Torabinejad M (1998) Cellular response to Mineral Trioxide Aggregate. *Journal of Endodontics* **24**, 543–7.
- Liu Y, Cotgreave I, Atzori L, Grafstrom RC (1992) The mechanism of Hg²⁺ toxicity in cultured human oral fibroblasts: the involvement of cellular thiols. *Chemico-Biological Interactions* **85**, 69–78.
- Mitchell PJ, Pitt Ford TR, Torabinejad M, McDonald F (1999) Osteoblast biocompatibility of mineral trioxide aggregate. *Biomaterials* **20**, 167–73.
- Osorio RM, Hefti A, Vertucci FJ, Shawley AL (1998) Cytotoxicity of endodontic materials. *Journal of Endodontics* **24**, 91–6.
- Peltola M, Salo T, Oikarinen K (1992) Toxic effects of various retrograde root filling materials on gingival fibroblasts and rat sarcoma cells. *Endodontics and Dental Traumatology* **8**, 120–4.
- Rudnick DA, Perlmutter DH, Muglia LJ (2001) Prostaglandins are required for CREB activation and cellular proliferation during liver regeneration. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 8885–90.
- Sauer SK, Schäfer D, Kress M, Reeh PW (1998) Stimulated prostaglandin E₂ release from rat skin, *in vitro*. *Life Sciences* **62**, 2045–55.
- Savla U, Appel HJ, Sporn PH, Waters CM (2001) Prostaglandin E(2) regulates wound closure in airway epithelium. *American Journal of Physiology: Lung Cellular and Molecular Physiology* **280**, L421–31.
- Schäfer D, Lindenthal U, Wagner M, Bölskei PL, Baenkler HK (1996) Effect of prostaglandin E₂ on eicosanoid release by human bronchial biopsy specimens from normal and inflamed mucosa. *Thorax* **51**, 919–23.
- Schäfer D, Schmid M, Göde UC, Baenkler HW (1999) Dynamics of eicosanoids in peripheral blood cells during bronchial provocation in aspirin-intolerant asthmatics. *European Respiratory Journal* **13**, 638–46.
- Shimauchi H, Takayama S, Miki Y, Okad H (1997) The change of periapical exudate prostaglandin E₂ levels during root canal treatment. *Journal of Endodontics* **23**, 755–8.
- Tai KW, Chang YC (2000) Cytotoxicity evaluation of perforation repair materials on human periodontal ligament cells *in vitro*. *Journal of Endodontics* **26**, 395–7.
- Takayama S, Miki Y, Shimauchi H, Okada H (1996) Relationship between prostaglandin E₂ concentrations in periapical exudates from root canals and clinical findings of periapical periodontitis. *Journal of Endodontics* **22**, 677–80.
- Torabinejad M, Hong CU, Pitt-Ford TR, Kettering JD (1995) Cytotoxicity of four root end filling materials. *Journal of Endodontics* **21**, 489–92.
- Wang RR, Li Y (1998) *In vitro* evaluation of biocompatibility of experimental titanium alloys for dental restorations. *Journal of Prosthetic Dentistry* **80**, 495–500.
- Wang JY, Tsukayama DT, Wicklund BH, Gustilo RB (1996) Inhibition of T and B cell proliferation by titanium, cobalt, and chromium: role of IL-2 and IL-6. *Journal of Biomedical Materials Research* **32**, 655–61.
- Wataha JC, Malcolm CT, Hanks CT (1995) Correlation between cytotoxicity and the elements released by dental casting alloys. *International Journal of Prosthodontics* **8**, 9–14.
- Willershausen B, Briseño Marroquin B, Schäfer D, Schulze R (2000) Cytotoxicity of root canal filling materials to three different human cell lines. *Journal of Endodontics* **26**, 703–7.
- Zhu Q, Haglund R, Safavi KE, Sponberg LS (2000) Adhesion of human osteoblasts on root-end filling materials. *Journal of Endodontics* **26**, 404–6.